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A kinetic microplate method for quantifying the antibacterial properties of biological fluids

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Abstract

The microplate assay for measuring antibacterial activity was adapted by incorporating a known concentration range of gentamicin as an internal standard. *Staphylococcus aureus* subsp. *aureus* strain ATCC 6538P, *Escherichia coli* ATCC 25922, and *Lactobacillus* spp. were used as target organisms, although other indicator organisms and antibiotics can be examined. Assay range and sensitivity were dependent on the species and density of indicator organism, and conditions (e.g., type, concentration, and pH of growth medium). Plotting the area under the growth curve (AUGC) versus gentamicin concentration (log transformed) yielded a linear curve that was used to quantify in units of gentamicin the antibacterial activity of a secretory fluid (SCF; pancreatic juice) and for comparisons of samples collected at different times, analysed on different days, and from different studies. This adaptation of the microtiter broth method will be useful for investigating man-made compounds, and the antibacterial activity of secretory fluids and the influences of age, diet, and health status.

Keywords: Antibacterial activity; Broth dilution; Microplate assay; Pancreatic juice

1. Introduction

The increasing number of bacterial and fungal strains that have developed resistance to classical antibiotics has intensified the search for new antibiotic compounds. Some of the newer discoveries include the numerous antimicrobial peptides produced by animals, plants, and microorganisms (Lambert et al., 2001; Meylears et al., 2002; Vizioli and Salzet, 2002; von Horsten et al., 2002).

Antibacterial activity has been reported for several secretory fluids (SCFs) of animals, including pancreatic juice, saliva, tears, and respiratory fluid (Diamond et al., 1991; Pierzynowski et al., 1993; Mahida et al., 1997; Huttner and Bevins, 1999; Travis et al., 1999). Of specific interest are the antibacterial activities of gastrointestinal SCFs against pathogens already resident in the gastrointestinal tract, in food, and associated with animals destined for human consumption.

Standardised methods for measuring activity are needed to define the spectrum and characteristics of antibacterial activity in SCF and to explore the potential mechanisms of induction through diet modulation or by using specific inducers. Traditionally, antibac-

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terial activity of SCF has been measured using agar diffusion assays. This approach involves exposing target organisms embedded in an agar plate to SCF using impregnated sterile disks of filter paper, or by placing the SCF directly on the surface or into preformed wells (Pilet and Toma, 1969; Fuchs et al., 1995). After an appropriate period of exposure, the size of the zone of growth inhibition around the SCF delivery area is measured and considered as the indicator of potency. Agar diffusion assays can be costly, time consuming, and the measurements tend to be more qualitative than quantitative. A further limitation is that the majority of antimicrobial peptides in SCFs are cationic (Ganz and Lehrer, 1998; Huttner and Bevins, 1999; Hancock and Diamond, 2000) and can interact with the net negative moieties in agar (Kunin and Edmondson, 1968), reducing diffusion and activity.

Alternatives to diffusion assays have been developed to reduce time and costs, address limitations, and increase sensitivity for detection of antibacterial activity in SCFs. Notable is the adaptation of the broth dilution method for a microplate reader format, which allows investigators to rapidly and simultaneously analyse small volumes of multiple samples. Antibacterial activity is evaluated based on the length of the lag phase, the slope of the exponential growth phase, and the area under the growth curve (AUGC), and has been used to examine antibacterial activity of pancreatic juice (Minelli et al., 1996). Still, measuring the effects and minimum inhibitory concentration (MIC) of antimicrobial peptides has remained a challenge for the microtiter approach, especially when comparative information is required, or the observed responses are non-linear. A modified Gompertz function has been used to fit the data, from which a more exact MIC value can be obtained (Lambert and Pearson, 2000), whereas susceptibility results have been determined by using either classical Chick–Watson (CW) log–linear disinfection kinetics or the empirical, non-linear time Hom model (Lambert et al., 1999).

This paper describes a microplate assay that incorporates an internal standard of a known concentration range of gentamicin. The internal standard addresses the above limitations, and permits direct comparisons of data obtained at different times, using different assay conditions, and SCF originating from different tissues or organs. This approach was used to quantify the antibacterial activity in a SCF (exocrine pancreatic secretion) in units of gentamicin, and allowed for comparisons among samples that were analysed on different days, from different animals and studies, and for various bacterial indicators.

2. Materials and methods

2.1. Reagents

A phosphate-buffered saline (PBS) was used as a diluent and was prepared with (in mmol/l) NaCl (130.90), Na_2HPO_4 (8.94), NaH_2PO_4 (0.83), and KH_2PO_4 (1.55), with pH = 7.2 ± 0.1. The growth medium was a soybean-casein digest (pH 7.3 ± 0.2) containing Trypticase® soy broth (TSB; Becton Dickinson Cockeysville, MD). The solutions were prepared with glass distilled water and were autoclaved (121 °C and 15 psi; 15 min) prior to use.

A series of gentamicin standards was prepared using sterile PBS to serially dilute (two-fold) a stock solution of 1280 μg/ml sterile water gentamicin sulfate (Sigma; St. Louis, MO). Samples of exocrine pancreatic secretion collected from pigs with surgically implanted reentrant pancreatic duct catheters (Pierzynowski et al., 1993) were used as a model SCF. The antibiotic and SCF were filter sterilized (0.2 μm; Supor® Acrodisc, Gelman Sciences, Ann Arbor, MI) prior to use.

2.2. Materials

The assays were performed on microtiter plates (Nunc-Immuno™ Maxisorp; Nalge NUNC International, Roskilde, Denmark) that were blocked with casein (Casamino acids, technical grade; Difco Laboratories, Detroit, MI). The plates were sealed with breathable membranes (Nalge NUNC International) before they were placed in a microtiter plate reader (SpectraMax 384 Plus, Molecular Devices, Sunnyvale, CA).

2.3. Preparation of cells

Staphylococcus aureus subsp. *aureus* strain (ATCC® 6538P) was used as the principal indicator organism, and was grown for 24 h on blood agar plates (5% sheep red blood cells, and tryptic soy agar

(TSA)) at 37 °C. The cells were suspended in PBS to an optical density of 0.6 at 595 nm (OD_{595}), and diluted with TSB to obtain a density of 7×10^5 colony forming units per ml (CFU/ml), which was confirmed by plate counts.

The assay was also evaluated using *Escherichia coli* (ATCC 25922) and an isolate of *Lactobacillus* spp. from pig faeces as target organisms. The *E. coli* and *Lactobacillus* spp. were propagated and diluted following the protocol for *S. aureus*.

2.4. Microtiter broth method and incorporation of a gentamicin standard

Growth inhibition was measured using a modification of a previously described microtiter broth method (du Toit and Rautenbach, 2000). Briefly, the microtiter plates were blocked with 0.5% w/v casein in PBS for 1 h, air dried, and sterilized under ultraviolet light (12 h; short and long wavelengths).

The first six wells contained TSB (50 μl) and sterile PBS (50 μl) and were used as sterility controls and blanks. The remaining two wells in the column were filled with TSB (50 μl) and 50 μl of a 2.5 μg/ml gentamicin solution to ensure sterility of the standards. The remaining wells used for an assay contained 50 μl of the bacterial suspension and either 50 μl of sterile PBS (growth controls), pancreatic secretion (test samples), or a series of gentamicin solutions with concentrations ranging from 2.5 to 0.04 μg/ml. The sealed plates were incubated at 37 °C inside the microtiter plate reader and the OD_{595} of each well was measured every 10 min for 24 h. The plates were automatically shaken for 15 s prior to each OD reading.

The minimum inhibitory concentration (MIC) of gentamicin for *S. aureus* was determined by adding 50 μl of each concentration of gentamicin (0.0006–1280 μg/ml) to duplicate wells with 50 μl of the bacterial suspension. The gentamicin MIC for *S. aureus* (2.5 μg/ml) was the highest concentration used on each plate for preparing the standard curve. The same approach was used to determine the gentamicin MIC for *E. coli* and the *Lactobacillus* spp. (Fig. 3).

2.5. Data processing

The area under the growth curve (AUGC) was calculated and integrated using SoftMax Pro 4.0 (Molecular Devices). Potential differences in the OD_{595} among individual wells were accounted for by subtracting the initial OD_{595} from the values measured during the 24-h incubation, yielding net OD_{595} changes for each well.

Linear regression analysis was performed using the semi-log curve fit option in SoftMax and the range of standards over which there was an inverse relationship between concentration of gentamicin (log transformed) and bacterial growth based on AUGC. Standards that were outside of the linear range were masked (Fig. 2).

The equation used for the line was:

$$Y = A + B \times \log(X)$$

where Y is the AUGC, A is the y-intercept, B is the slope of the log transformed data, and X is the gentamicin concentration. The coefficients were used to convert the AUGCs for the pancreatic juice samples into gentamicin concentrations.

3. Results and discussion

3.1. Development of the standard curve

The present study used *S. aureus* as the indicator organism because it is an opportunistic pathogen that is absent from the normal flora of the gut (Savage, 1977, 1981) and has previously been used for studying antibacterial activity of pancreatic secretion (Pierzynowski et al., 1992, 1993). Gentamicin was selected as the antibiotic standard because it inhibits protein synthesis in a bactericidal manner in both Gram-negative and Gram-positive bacteria (Brenciaglia et al., 1996), including *S. aureus*, and can therefore be used for studying disparate types of bacteria.

Over the range of 2.5 μg gentamicin/ml (the MIC) to 0.313 μg/ml, there was a direct relationship between gentamicin concentration and the lag phase for growth of *S. aureus* (Fig. 1). Lower gentamicin concentrations did not inhibit growth. Plotting the AUGC as a function of gentamicin concentration yielded similar findings (Fig. 2). The linear relationship that resulted when the AUGC data for *S. aureus* were plotted as a function of log transformed genta-

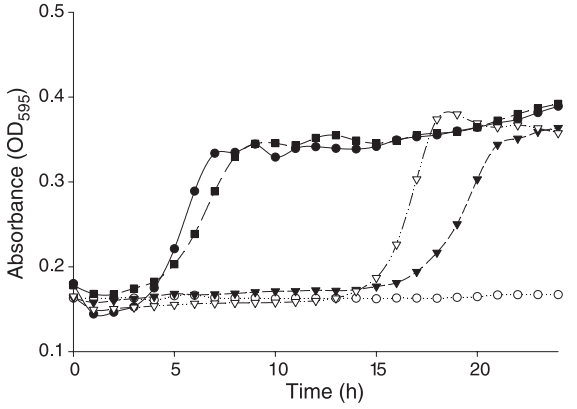

Fig. 1. Representative growth curves (change in OD_{595} over time) for *S. aureus* exposed to gentamicin concentrations of 0 (●), 0.313 (■), 0.625 (▽), 1.25 (▼), and 2.5 (○) μg/ml. The MIC value shown on the graph is 2.5 μg/ml.

micin concentrations over the working range of the assay (2.5 to 0.313 μg/ml; values below 0.313 μg/ml were masked) was used as the standard curve (Fig. 3). Standard curves prepared for *E. coli* ATCC 25922 and *Lactobacillus* spp. (Fig. 3) indicate that the relationship between log gentamicin concentration and AUGC varies among indicator organisms. Therefore, the working range of the assay must be defined when different organisms are used, and the volumes or antibacterial activity of unknowns must yield AUGCs that are within the range.

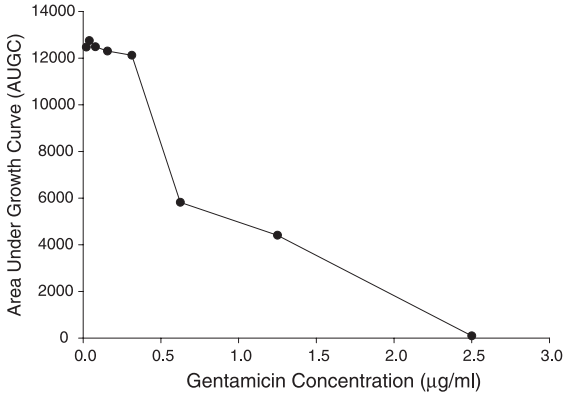

Fig. 2. The area under the growth curves for *S. aureus* in the presence of gentamicin concentrations from 2.0×10^{-2} to 2.5 μg/ml. Note that the area under the growth curve does not increase further when a gentamicin concentration of 0.313 μg/ml or less is used.

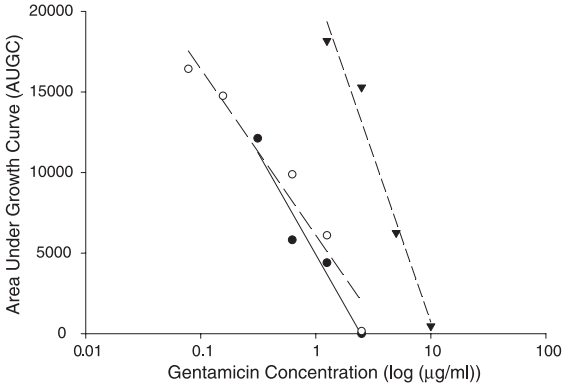

Fig. 3. Representative linear standard curves for growth of *S. aureus* (●, solid line; $r^2 = 0.95$), *E. coli* (▼, short dash; $r^2 = 0.97$), and *Lactobacillus* spp. (○, long dash; $r^2 = 0.95$) as functions of gentamicin concentration (log transformed).

3.2. Antibacterial activity of exocrine pancreatic secretion

The present adaptation of the microtiter broth method has proven useful for quantifying the antibacterial activity in pancreatic secretion. Specifically, the ability to convert AUGC data to units of gentamicin activity has allowed us to quantify the magnitude of variation for antibacterial activities among samples of pancreatic secretion that were collected from the same animal (Fig. 4) or from different animals (data not shown). Moreover, the values did not vary when the same sample was assayed on different plates or days, or when the target bacteria (*S. aureus*) originated from

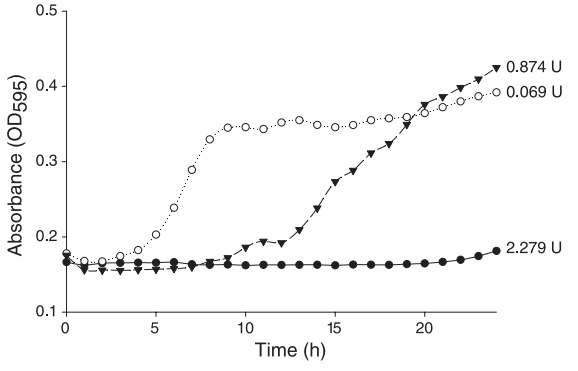

Fig. 4. Representative growth curves for *S. aureus* exposed to different samples of pancreatic juice collected from a pig. Antibacterial activity as units of gentamicin (2.279 (●), 0.874 (▼), and 0.069 (○) μg/ml) was calculated from the standard curve.

different cultures, or were used at different ages and densities. In addition, two-fold dilutions of pancreatic juice samples resulted in corresponding decreases in antibacterial activity (data not shown).

The present findings show that the incorporation of an internal standard into the microtiter broth method allows investigators to quantify antibacterial activities of secretory fluids and evaluate the variation among sources and in response to diet and feeding state, age, health status, and environmental conditions. However, the dynamic range for the assay must be defined for the selected conditions and equipment, target organism, selected antibiotic, and the SCF of interest. Therefore, investigators will need to validate and optimize the assay before quantifying the antibacterial activity of SCF against various bacteria using alternative antibiotic standards.

Table 1
Factors to take into consideration when designing an assay for measuring antibacterial activity in SCF

Consideration	Factor	Comment
Selection of target organism and assay conditions	Aerotolerance	Limited to O_2 environment
	Nutrient requirements	Some bacteria require specific growth factors
	Growth patterns in broth	Clumping may be a factor
	Broth concentration	Affects lag time
	Temperature	Range = 4–45 °C
	pH	Must meet bacteria and SCF requirements
	Sample: bacteria	Flexible depending on effectiveness of sample
Selection of antibiotic	Mode of action	Bactericidal Bacteriostatic Membrane directed Inhibits protein synthesis

4. Conclusions

The incorporation of an internal standard increases the utility of the microtiter broth dilution method and the possible applications. The present results with *S. aureus*, *E. coli*, and *Lactobacillus* spp. demonstrate that the assay is compatible for studying how antimicrobial compounds affect the diversity of organisms. Although not verified, it is expected that gentamicin can be substituted with other antibiotics, depending on the target organism, potency of the sample, and the desired mode of bacterial inhibition.

The assay is not limited to TSB as the growth medium. We have recorded comparable results with brain heart infusion (data not presented). However, there are several considerations before using alternative media, including the nutritional requirements of the indicator organism (Table 1). The media can influence antibacterial activity of some SCF. For example, media with a pH outside the range of 7.2–8.0 will reduce or inactivate the antibacterial activity in pancreatic secretion (our unpublished data). Assay sensitivity is also responsive to nutrient concentrations. For example, using double strength TSB (1 X in final assay volume after addition of standards, pancreatic secretion, or PBS) decreased the lag phase and reduced assay sensitivity compared to when the TSB was 0.5 X in the final sample volume (data not shown).

The present modification of the microtiter broth dilution method allows investigators to quantify and express antibacterial activity in SCFs as units of a known antibiotic. The approach does not suffer from the many limitations associated with the use of gel media and allows for the immediate interaction between the antibacterial compounds and indicator organism. Moreover, the use of different species of bacteria, antibiotics, and growth conditions will allow investigators to expand the dynamic range and sensitivity of the assay. Although only aerotolerant bacteria (facultative or obligate aerobes) have been studied to date, it may be possible to measure antibacterial activity under anaerobic conditions by adding Oxyrase (Oxyrase, Mansfield, OH) to the test medium (Wiggs et al., 2000) or by placing the plate reader in an anaerobic chamber. Alternatively, multiple plates could be incubated anaerobically and end-point OD recorded at selected time intervals to obtain estimates of lag phase duration, rate of exponential growth, and AUGC.

Notation

AUGC area under the growth curve
CFU/ml colony forming units per millilitre
ELISA enzyme-linked immunosorbent assay
MIC minimum inhibitory concentration
OD optical density
PBS phosphate-buffered saline

SCF secretory fluid
TSA tryptic soy agar
TSB Trypticase soy broth

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